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Emergence of Multidrug-Resistant Pneumococcal Serotype 35B among Children in the United States

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ABSTRACT *Streptococcus pneumoniae* serotype 35B is a nonvaccine serotype associated with high rates of penicillin nonsusceptibility. An increase in the proportion of multidrug-resistant (MDR) 35B isolates has recently been reported. The genetic events contributing to the emergence of MDR serotype 35B are unknown. The sequence type (ST) composition of 78 serotype 35B isolates obtained from pediatric patients with invasive pneumococcal disease from 1994 to 2014 and 48 isolates from pediatric patients with otitis media (noninvasive) from 2011 to 2014 was characterized by multilocus sequence typing (MLST). The most common STs were ST558 (69.2%), ST156 (10.3%), and ST452 (3.8%). Two major clonal complexes (CC), CC558 and CC156, were identified by eBURST analysis. Overall, 91% (71/78) of isolates were penicillin nonsusceptible and 16.7% (13/78) were MDR. Among all invasive serotype 35B isolates, MDR isolates increased significantly, from 2.9% (1/35) to 27.9% (12/43) ($P = 0.004$), after the 13-valent pneumococcal conjugate vaccine (PCV13) was introduced. All CC156 isolates were identified after the introduction of PCV13 (0/35 [0%] before versus 9/43 [20.9%] after; $P = 0.003$) and were MDR. All CC156 isolates had similar antimicrobial susceptibility patterns; in contrast, high variability in antimicrobial susceptibility was observed among CC558 isolates. The distributions of CC558 and CC156 among invasive and noninvasive isolates were not different. The increased prevalence of MDR serotype 35B after the introduction of PCV13 was directly associated with the emergence of ST156. Genotyping suggests that capsular switching has occurred between MDR vaccine serotypes belonging to ST156 (e.g., 9V, 14, and 19A) and serotype 35B.

KEYWORDS *Streptococcus pneumoniae*, pneumococcal disease, pneumococcal vaccine, serotype 35B, multidrug resistance, otitis media, pneumococcus

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After the introduction of the 13-valent pneumococcal conjugate vaccine (PCV13) (Prevnar 13; Pfizer) in 2010 in the United States, a significant decline in the incidence of invasive pneumococcal disease (IPD) among children was observed (1, 2). PCV13 has also had a positive impact on noninvasive disease, such as sinusitis and otitis media (3, 4). However, as a result of vaccine selection pressure, non-PCV13 serotypes are now predominant (5–8). Of particular interest is the expansion of serotype 35B among nasopharyngeal and noninvasive disease samples from children and adults, which was reported before the introduction of PCV13 and increased significantly post-PCV13 (4–7, 9). IPD associated with serotype 35B has also increased further after the introduction of PCV13; however, few studies exist on this topic (6, 8). In 2014,

Streptococcus pneumoniae serotype 35B became the most common serotype causing IPD in our United States Pediatric Multicenter Pneumococcal Surveillance Group.

Serotype 35B is not included in PCV13 or the 23-valent pneumococcal polysaccharide vaccine (Pneumovax23; Merck). The increasing prevalence of serotype 35B is worrisome because it has long been associated with penicillin nonsusceptibility (10) and, most recently, with multidrug resistance (MDR) in the United States (6, 11). In 2013, serotype 35B represented 59.9% of multidrug-nonsusceptible nonvaccine serotypes among IPD isolates from all age groups in the United States (11). In Europe, other nonvaccine serotypes predominate; however, a moderate increase in serotype 35B IPD among adults and children has been described (12, 13). Nevertheless, serotype 35B has been associated with the highest risk for death due to IPD (relative risk [RR], 4.98; 95% confidence interval [CI], 2.49 to 9.95) among all serotypes affecting adults and children in Europe (13).

The genetic events contributing to the emergence of serotype 35B following the introduction of PCV13 are unknown. Horizontal genetic exchange plays a critical role in the evolution of *S. pneumoniae* and provides an extraordinary ability to evade environmental stressors like vaccines (14–16). Sequence type (ST) analysis of pneumococcal isolates in the 7-valent pneumococcal conjugate vaccine (PCV7) era suggested that expansion of preexisting STs, the appearance of new STs, and capsular switching are some of the evolutionary pneumococcal responses observed in previous emergent serotypes (17–21). Capsular switching occurs when a strain of *S. pneumoniae* takes the capsular type of another strain by the transfer of the capsular locus (*cps*) genes (22). As a result, novel combinations of serotype and ST are generated which allow established circulating clones to adapt to the postvaccine environment by switching their serotype to a nonvaccine serotype (21). Capsular switching may include recombination of *cps* flanking regions like penicillin binding protein-encoding genes *pbp1A* and *pbp2X*, which can be altered and, thus, lead to penicillin resistance (23, 24).

The expansion of serotype 35B presents a unique opportunity to study the genetic adaptation of *S. pneumoniae* and its antibiotic resistance as it emerges. The aim of this study was to characterize the molecular epidemiology of serotype 35B isolates among pediatric patients with IPD in the United States over a period of 21 years, focusing on ST changes that may have contributed to the emergence of serotype 35B and their association with antibiotic resistance patterns. We also aimed to characterize the disease potential of STs within serotype 35B (noninvasive versus invasive).

RESULTS

Demographic characteristics and clinical presentation. Seventy-eight invasive serotype 35B isolates were identified out of 5,420 IPD isolates from 1994 to 2014. The median age of the 78 subjects was 19.6 months (interquartile range [IQR], 9.2 to 72.8), and 65.4% (51/78) were male. The race/ethnicity distribution was 39.7% (31/78) white, 26.9% (21/78) black, 25.6% (20/78) Hispanic, and 7.7% (6/78) other. Forty subjects (51.3%) had an underlying medical condition or comorbidity. The distributions of the demographic characteristics listed above were similar in the pre- and post-PCV13 eras. The immunization status was evaluated in patients identified from 2000 to 2014 ($n = 75$). Immunization status was not available for 11 subjects (14.7%); of the other 64, 18 (28.1%) were unimmunized and 46 (71.9%) had received at least one dose of PCV7 or PCV13.

The most common clinical syndrome was bacteremia without focus ($n = 34$; 43.6%), followed by meningitis ($n = 16$; 20.5%), pneumonia ($n = 8$; 10.3%), osteoarticular infection ($n = 7$; 8.9%), mastoiditis ($n = 5$; 6.4%), and others (peritonitis, ventriculitis, endophthalmitis, sinusitis with intracranial extension, skin and soft tissue infection with bacteremia, and dacryocystitis). The distribution of clinical syndromes also remained unchanged after the introduction of PCV13.

Sequence type characterization. Of the 78 invasive serotype 35B isolates, 44.9% (35/78) were identified in the pre-PCV13 era and 55.1% (43/78) in the post-PCV13 era.

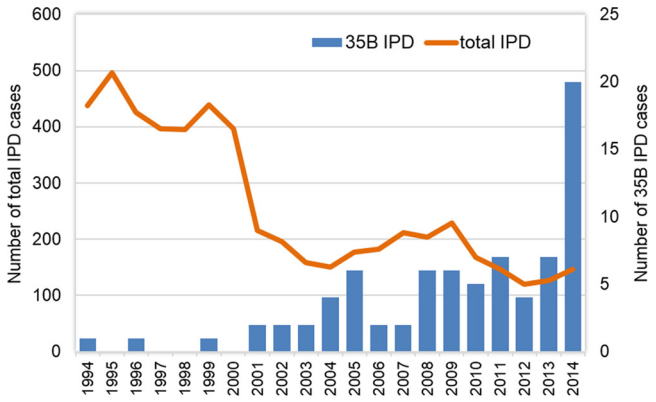

FIG 1 Number of serotype 35B invasive isolates by year from 1994 to 2014.

The number of invasive serotype 35B isolates increased significantly ($P < 0.001$) over time in proportion to all invasive isolates (Fig. 1).

A total of 15 different STs were identified; 7 have not been previously described. The allelic profiles of the 15 STs are shown in Table S1 in the supplemental material. The most common STs were ST558 ($n = 54$; 69.2%), ST156 ($n = 8$; 10.3%), and ST452 ($n = 3$; 3.8%). Of the other STs, 9 represent single-locus variants (SLVs) of ST558, 1 represents a double-locus variant (DLV) of ST558, and 1 represents an SLV of ST156. The eBURST analysis (Fig. 2) divided the STs into two clonal complexes (CCs) (CC558, $n = 65$, and CC156, $n = 9$) and two singletons (ST452, $n = 3$, and ST198, $n = 1$).

Overall, the demographic characteristics and clinical syndromes were similar for CC558 and CC156 (Table 1). Patients with isolates characterized as CC156 were younger

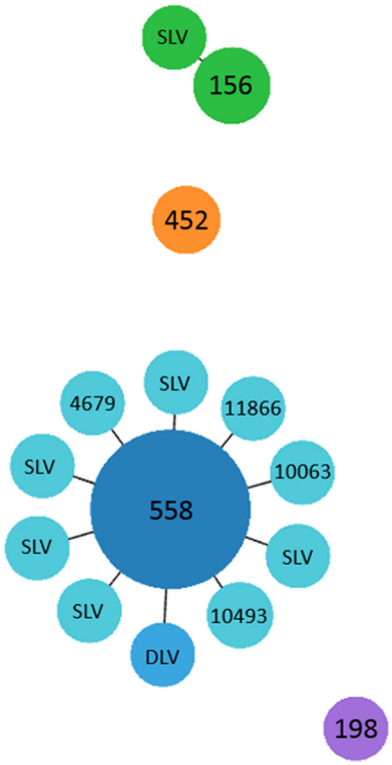

FIG 2 eBURST analysis of 78 invasive serotype 35B isolates. Each sequence type (ST) is represented by a circle or node. The size of the circle corresponds to the number of isolates belonging to that ST. STs sharing 6 alleles (single-locus variant [SLV]) or 5 alleles (double-locus variant [DLV]) are connected by a black line. New ST designations are pending.

TABLE 1 Demographics and clinical syndromes per clonal complex of invasive serotype 35B isolates from 1994 to 2014

Characteristic	No. (%) of patients (or value as indicated) with isolates from clonal complex:		*P* value[b]
	558 (*n* = 65)	156 (*n* = 9)	
Age (mos) (IQR[a])	33.3 (9.8–86.4)	10.0 (5.4–41.9)	0.08
Male	41 (63.1)	6 (66.7)	1
Race/ethnicity			
White	28 (43.1)	3 (33.3)	0.7
Black	13 (20)	5 (55.6)	**0.03**
Hispanic	18 (27.7)	1 (11.1)	0.4
Other	0	6 (9.2)	
Comorbidity	35 (53.8)	5 (55.6)	1
Clinical syndrome			
Bacteremia	28 (43.1)	6 (66.7)	0.3
Meningitis	13 (20)	0	0.3
Pneumonia	6 (9.2)	2 (22.2)	0.2
Osteoarticular infection	5 (7.7)	1 (11.1)	0.5
Mastoiditis	5 (7.7)	0	1
Other	8 (12.3)	0	

[a]IQR, interquartile range.
[b]Statistically significant values are in boldface.

than patients with CC558 isolates, but this difference was not statistically significant. A greater proportion of CC156 isolates than of CC558 isolates were from black patients ($P = 0.03$).

All CC156 isolates were identified after the introduction of PCV13 (0/35 [0%] before versus 9/43 [20.9%] after; $P = 0.003$), as shown by the results in Fig. 3. Serotype 35B^{ST156} was identified for the first time in 2011 in our surveillance study. After the introduction of PCV13, the proportion of CC558 isolates from among all serotype 35B isolates decreased from 97.1% (34/35) to 72.1% (31/43) ($P = 0.004$). Within CC558, more SLVs and DLVs were observed over the period from 2010 to 2014; however, this observation did not reach statistical significance (3/35 [8.6%] versus 8/43 [18.6%]; $P = 0.3$).

Antibiotic susceptibility patterns and sequence types. Overall, 91% (71/78) of serotype 35B isolates were penicillin nonsusceptible, 7.7% (6/78) were ceftriaxone

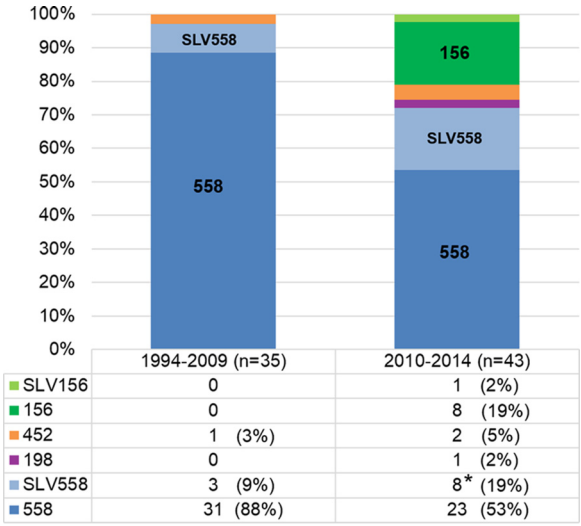

FIG 3 Sequence type (ST) distribution before and after the introduction of PCV13. *, category includes a double-locus variant.

TABLE 2 Antibiotic susceptibilities of invasive serotype 35B isolates before and after the introduction of PCV13

Vaccine era	Median MIC (μg/ml) (IQR[a]) of:		No. (%) of isolates that were:					
			Nonsusceptible to:		Resistant to:			
	Penicillin	Ceftriaxone	Penicillin (MIC of ≥0.12 μg/ml)	Ceftriaxone (MIC of ≥1 μg/ml)	Erythromycin	Clindamycin	TMP-SMX[b]	Multidrug resistant
Pre-PCV13 (n = 35 isolates)	1 (0.5–1)	0.5 (0.25–0.5)	34 (97.1)	3 (8.6)	8 (22.9)	0	2 (5.7)	1 (2.9)
Post-PCV13 (n = 43 isolates)	1 (0.25–1)	0.25 (0.12–0.5)	37 (86)	3 (7)	28 (65.1)	1 (2.3)	15 (34.9)	12 (27.9)
P value[c]	0.4	**0.009**	0.1	1	**<0.001**	1	**0.002**	**0.004**

[a]IQR, interquartile range.
[b]TMP-SMX: trimethoprim-sulfamethoxazole.
[c]Statistically significant values are in boldface.

nonsusceptible, 46.2% (36/78) were erythromycin resistant, 1.3% (1/78) were clindamycin resistant, 21.8% (17/78) were trimethoprim-sulfamethoxazole (TMP-SMX) resistant, and 16.7% (13/78) were considered MDR.

Antibiotic susceptibility patterns were compared before and after the introduction of PCV13 (Table 2). There was no change in the proportion of penicillin-nonsusceptible, ceftriaxone-nonsusceptible, or clindamycin-resistant isolates after the introduction of PCV13. On the other hand, erythromycin- and TMP-SMX-resistant isolates, as well as MDR isolates, increased significantly after the introduction of PCV13. In this study, the first MDR serotype 35B isolate was identified in 2009 and was characterized as ST558. Figure S1 in the supplemental material shows the percentages of serotype 35B isolates by specific penicillin and ceftriaxone MICs and vaccine eras.

The antibiotic susceptibility patterns of CC558 and CC156 were also compared (Table 3). Even though the penicillin MIC IQR was slightly higher for CC156 isolates, the overall penicillin MIC distributions and the proportions of penicillin-nonsusceptible isolates were not statistically different between CC558 and CC156 isolates. The ceftriaxone MIC distribution was statistically higher among CC156 than CC558 isolates. Figure S2 in the supplemental material shows the percentages of serotype 35B isolates by specific penicillin and ceftriaxone MICs and CCs. The distributions of penicillin and ceftriaxone MICs among all STs are shown in Fig. 4A. All of the CC156 isolates were resistant to erythromycin and TMP-SMX and were identified as MDR, which differed significantly from CC558 isolates. The few cases of MDR isolates belonging to CC558 were ST558 and not a newly discovered SLV or DLV (Fig. 4B).

Disease potential of sequence types. Invasive and noninvasive isolates were used to compare the disease potential of CCs. Forty-eight noninvasive serotype 35B isolates out of 473 noninvasive pneumococcal isolates were identified from 2011 to 2014. These isolates were compared to 38 invasive serotype 35B isolates identified during the same time period. Table S2 in the supplemental material shows the demographic characteristics of patients with invasive and noninvasive serotype 35B isolates. The median age in both groups was 15.6 months. Patients with invasive disease had a higher percent-

TABLE 3 Antimicrobial susceptibility by clonal complex of invasive serotype 35B isolates from 1994 to 2014

Clonal complex	Median MIC (μg/ml) (IQR[a]) of:		No. (%) of isolates that were:					
			Nonsusceptible to:		Resistant to:			
	Penicillin	Ceftriaxone	Penicillin (≥0.12 μg/ml)	Ceftriaxone (≥1 μg/ml)	Erythromycin	Clindamycin	TMP-SMX[b]	Multidrug resistant
558 (n = 65 isolates)	1 (0.5–1)	0.5 (0.25–0.5)	62 (95.4)	3 (4.6)	27 (41.5)	1 (1.5)	8 (12.3)	4 (6.2)
156 (n = 9 isolates)	1 (0.75–2)	0.5 (0.37–1)	9 (100)	3 (33.3)	9 (100)	0	9 (100)	9 (100)
P value[c]	0.14	**0.03**	1	**0.02**	**0.001**	1	**<0.001**	**<0.001**

[a]IQR, interquartile range.
[b]TMP-SMX, trimethoprim-sulfamethoxazole.
[c]Statistically significant values are in boldface.

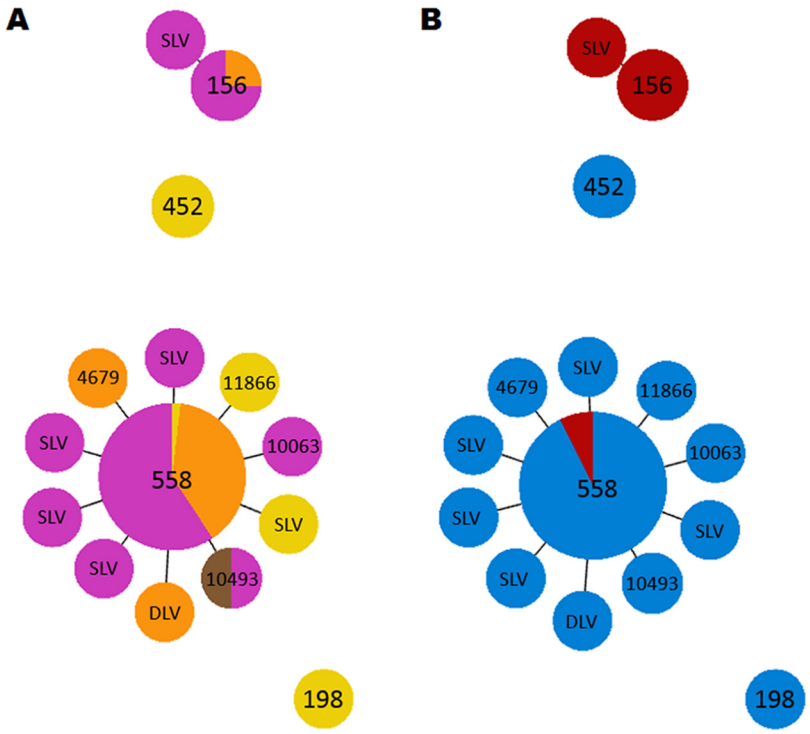

FIG 4 eBURST analysis of penicillin MIC distribution and multidrug-resistant serotype 35B isolates. (A) Penicillin MIC distribution among serotype 35B sequence types (STs). Yellow, 0.008 to 0.06 μg/ml; orange, 0.12 to 0.5 μg/ml; fuchsia, 1 to 2 μg/ml; brown, $\geq$4 μg/ml. (B) Distribution of multidrug-resistant (MDR) isolates among serotype 35B STs. Blue, non-MDR isolates; red, MDR isolates. New ST designations are pending.

age of comorbidity than patients with noninvasive disease (21/38 [55.3%] versus 7/48 [14.6%]; $P < 0.001$).

Among the 48 noninvasive isolates, 9 different STs were identified, including 2 STs that have not been previously described. The most common STs were ST558 ($n = 33$; 68.8%) and ST156 ($n = 8$; 16.7%). Table S3 in the supplemental material shows the allelic profiles of the 9 noninvasive STs. The eBURST analysis showed two CCs (CC558 and CC156) and one singleton (ST2414).

No CC was significantly associated with invasive or noninvasive disease. CC558 was identified in 26 invasive isolates (68.4%) and 38 noninvasive isolates (79.2%) ($P = 0.3$; odds ratio [OR], 0.6 [95% CI, 0.2 to 1.5]). CC156 was identified in 9 invasive isolates (23.7%) and in 9 noninvasive isolates (18.8%) ($P = 0.6$; OR, 1.3 [95% CI, 0.5 to 3.8]). The other STs included fewer than 5 isolates, and the ORs were not calculated. ST198 ($n = 1$) and ST452 ($n = 2$) were identified among invasive isolates. ST2414 ($n = 1$) was identified among noninvasive isolates. All meningitis cases were associated with CC558 and CC452; none were associated with CC156. Otherwise, CC558 and CC156 were responsible for bacteremia, pneumonia, and osteoarticular infections.

The antibiotic susceptibility pattern of the noninvasive isolates was similar to that of the invasive isolates, with the only exception being that all noninvasive 35B isolates were penicillin nonsusceptible, as shown by the data in Table S4 in the supplemental material. Thirty-one percent of isolates in each group were MDR.

DISCUSSION

In the present study, the number of cases of serotype 35B IPD increased considerably after the introduction of PCV13, which is consistent with recent reports (6, 8). The increase was directly related to the continued clonal expansion of CC558 and the emergence of CC156 among serotype 35B isolates. Other authors have described the predominance of CC558 among invasive serotype 35B isolates (8, 25, 26). However, no

other study has described the emergence of ST156 among invasive serotype 35B isolates after the introduction of PCV13 to the extent that has been observed in this study (0% before the introduction of PCV13 versus 20.9% after; $P = 0.003$). Preliminary data analysis of our 2015 noninvasive serotype 35B isolates shows a continued association of CC156 with this serotype past the period detailed in this communication (data not shown).

ST156 was first described in association with the widely disseminated MDR serotype 9V strain known as PMEN clone Spain^{9V}-3; however, it has also been associated with other serotypes, such as 14, 19F, and 19A (27, 28). In the United States, a recent study reported 2 capsular switching events that involved a serotype 35B donor strain (35B/ST558) and an ST156 recipient strain; the first occurred in 2009 and the second in 2012 (8). A study from Canada also identified a serotype 35B isolate that represented an SLV of ST156 (ST9346) in 2011 (25). The two studies reported 1 or 2 occurrences of ST156 or its SLV in association with serotype 35B (8, 25). Thus, ST156 did not contribute to the increased prevalence of serotype 35B in those studies. The discrepancy between the two previous studies and our study is likely related to the difference in the post-PCV13 years included. Six of the nine invasive CC156 isolates in our study were identified in 2014, while the other studies only included isolates up to 2013. Also, it is possible that the distribution of CCs among serotype 35B isolates varies geographically.

The emergence of ST156 among serotype 35B isolates after the introduction of PCV13 may represent a vaccine escape phenomenon in response to PCV13 selective pressure. CC156 is not closely related to CC558. In fact, the two complexes differ by multilocus sequence type (MLST) in all genetic loci tested. The strong association of ST156 with vaccine serotypes like 9V and 14 in the pre-PCV13 era suggests that ST156 has exchanged its *cps* encoding a vaccine serotype capsule with genes encoding a 35B capsule. In the United States, no evidence of capsular switching among serotype 35B isolates was reported before 2009 (8, 26, 27). Continuous surveillance will determine whether this vaccine escape recombinant (serotype 35B^{ST156}) will successfully spread.

High rates of penicillin nonsusceptibility (>90%) were observed among all serotype 35B isolates in our study, as has been described before (6, 10, 12, 29). Ceftriaxone nonsusceptibility represented <10% in our study; this percentage differs from an antimicrobial resistance surveillance program that reported 29.7% ceftriaxone nonsusceptibility among serotype 35B isolates obtained mainly from the East South Central and South Atlantic U.S. Census regions (30). In the present study, different rates of resistance to erythromycin and TMP-SMX were observed, but resistance to clindamycin was rare (1%). Higher penicillin and ceftriaxone MICs were observed among CC156 isolates than among CC558 isolates. A characteristic antibiotic susceptibility profile was identified for the CC156 isolates (penicillin nonsusceptible, erythromycin and TMP-SMX resistant, clindamycin susceptible, and variously ceftriaxone susceptible). The antibiotic susceptibility profiles for CC558 isolates varied.

After the introduction of PCV13, the proportion of MDR isolates among serotype 35B increased significantly in our study. Another study described that the proportion of MDR serotype 35B isolates increased from 0.4% to 6.4% ($P < 0.001$) from 2008 to 2012 in the United States (6). We found a clear association between the increase in multidrug resistance among serotype 35B isolates and the emergence of CC156. MDR was uncommon among CC558 isolates. In contrast, all invasive and noninvasive CC156 isolates were classified as MDR. This finding was related to erythromycin and TMP-SMX resistance. It is important to note that our MDR definition is based on penicillin nonsusceptibility (MIC of ≥0.12 μg/ml) for all our isolates. The necessary serum concentrations of penicillin typically can be achieved with correct dosages to successfully treat pneumococcal infections other than meningitis due to isolates with penicillin MICs of 0.12 to 2 μg/ml, and thus, the clinical implications of penicillin nonsusceptibility should be interpreted according to the clinical syndrome (31).

The clonal expansion of ST558 is the major contributor to the increasing prevalence of serotype 35B. However, in most recent years, the emergence of ST156 among serotype 35B isolates has also played a key role in the continued increase of this

serotype in our study population. This observation has some similarities to the expansion of serotype 19A after the introduction of PCV7. The initial increase in the prevalence of serotype 19A in the years following the introduction of PCV7 through 2005 was secondary to the clonal expansion of ST199, an ST previously associated with serotype 19A (32). In the period from 2006 to 2010, a clonal shift occurred toward MDR CC320, which became the major CC among serotype 19A isolates in the late 2000s and contributed to the dramatic expansion of serotype 19A among children (17, 19). Even though serotype 35B has become one of the most common serotypes causing disease in pediatric patients, the number of cases has not yet reached the magnitude of serotype 19A in the late 2000s. ST2414, found among our noninvasive isolates, is an SLV of ST63 (PMEN clone Sweden^{15A}-25) and has not been associated with serotype 35B. ST63 is associated with MDR serotypes like 15A, 19A, and 14 (33). Even though we only identified one ST2414 isolate, it potentially indicates the expansion of serotype 35B into other antibiotic-resistant lineages.

The distributions of the two major CCs (CC558 and CC156) among invasive and noninvasive isolates were similar. Our results on disease potential were not conclusive, likely secondary to the small number of isolates belonging to each of the two main CCs. However, serotype 35B has been associated with lower levels of invasiveness (OR, 0.4 [95% CI, 0.2 to 0.9]) than other serotypes (34). Possibly, the high rates of comorbidity (>50%) among patients with an invasive serotype 35B isolate in our study reflect the pathogenic potential of a normally low-pathogenic strain within susceptible populations. *In vitro* studies show that serotype 35B is capable of forming substantial amounts of biofilm, similar to 19F and 19A, which may facilitate nasopharyngeal colonization and mucosal disease (35). Pneumococcal virulence factors such as pili have been found among CC558 and CC156 isolates (25, 36, 37). These virulence factors facilitate colonization, and they are thought to contribute to invasiveness as well (38). The fact that pili are present within isolates of both CCs might provide them with similar opportunities for mucosal attachment and colonization.

In summary, serotype 35B has become an important serotype causing invasive and noninvasive disease in pediatric patients after the introduction of PCV13 in the United States. The increased prevalence of this serotype was associated with the clonal expansion and diversification of CC558 and the emergence of MDR CC156 among serotype 35B isolates. Genotyping suggests that serotype 35B^{ST156} may represent a vaccine escape recombinant resulting from capsular switching. The continuous emergence of serotype 35B over the next years could lead to a significant increase in IPD and to antibiotic resistance among pneumococcal isolates. Understanding the spread of MDR *S. pneumoniae* is also crucial for future guidance of empirical antibiotic use among pediatric patients with IPD and otitis media. Continuous surveillance is critical to further evaluate the impact of serotype 35B and its clonal switches in the pediatric population.

MATERIALS AND METHODS

Selection of pneumococcal isolates. The United States Pediatric Multicenter Pneumococcal Surveillance Group consists of 8 children's hospitals in the United States (Houston, TX; Pittsburgh, PA; Little Rock, AR; San Diego, CA; Los Angeles, CA; Chicago, IL; Columbus, OH; and Winston-Salem, NC) and has been collecting invasive pneumococcal isolates since 1993 and pneumococcal otitis media isolates since 2011. Through our prospective surveillance group, 6,035 pneumococcal isolates from pediatric patients have been collected from 1994 to 2014.

IPD caused by serotype 35B from 1 January 1994 to 31 December 2014 and cases of otitis media caused by serotype 35B from 1 January 2011 to 31 December 2014 were identified from our database. Subjects aged 0 to 18 years were included in this study. Invasive pneumococcal disease was defined as the isolation of *S. pneumoniae* from normally sterile sites (e.g., blood, cerebrospinal fluid, pleural fluid, bone, synovial fluid, etc.). Isolates from the middle ear of pediatric patients with otitis media (noninvasive samples) were obtained during myringotomy, pressure-equalizing-tube placement, or spontaneous drainage. Demographic and clinical information was abstracted from medical records. The study was approved by the institutional review boards of each of the participating hospitals.

All *S. pneumoniae* isolates are serotyped in the Infectious Diseases Research Laboratory at Texas Children's Hospital, Houston, TX, by the Quellung reaction using commercially available antisera (Statens Serum Institut, Copenhagen, Denmark, and Cedarlane Laboratories, Inc., Burlington, NC) (39).

Multilocus sequence typing. Multilocus sequence typing (MLST) was performed on extracted DNA as previously described (40). Bacterial DNA was extracted from fresh bacterial cultures using the

UltraClean microbial DNA isolation kit (Mo Bio, Carlsbad, CA). PCR amplification of the seven housekeeping gene fragments (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*) used in the pneumococcal MLST scheme was performed by using Platinum *Taq* DNA high-fidelity polymerase (Thermo Fisher Scientific, Inc., Waltham, MA). The primer pairs used for PCR amplification of the seven housekeeping gene fragments are available at http://pubmlst.org/spneumoniae/info/primers.shtml. The same primers were used for DNA sequencing. Purification of amplicons and sequencing in both forward and reverse directions were performed at an outside facility (Beckman Coulter Genomics, Danvers, MA).

The sequences obtained were queried in the online MLST database (http://pubmlst.org/spneumoniae/) to obtain distinct numerical allelic profiles and STs. All instances of unusual serotype-sequence type associations were reexamined by serotyping and/or genotyping. A previously undescribed serotype-sequence type association was considered a capsular switch. New alleles were submitted to the database curator, and designations are pending.

eBURST analysis. Genetic relatedness between isolates was inferred using the eBURST (based upon related sequence type) algorithm found at www.phyloviz.net (41). eBURST analysis groups STs into clonal complexes (CCs), clusters of closely related STs believed to have descended from the same founding ST, based on the number of differences between the allelic profiles.

Antibiotic susceptibility testing. Susceptibility to five antibiotics (penicillin, ceftriaxone, erythromycin, clindamycin, and trimethoprim-sulfamethoxazole [TMP-SMX]) often used to treat infections in pediatric patients was determined by obtaining the MICs of isolates. Antibiotic susceptibility testing was performed using the 2016 guidelines of the Clinical and Laboratory Standards Institute (CLSI) (42). MICs were determined by standard broth microdilution with Mueller-Hinton medium supplemented with 3% lysed horse blood. Penicillin nonsusceptibility was defined as a MIC of $\geq$0.12 μg/ml, and ceftriaxone nonsusceptibility as a MIC of $\geq$1 μg/ml. Resistance to erythromycin (MIC of $\geq$1 μg/ml), clindamycin (MIC of $\geq$1 μg/ml), and TMP-SMX (MIC $\geq$4/76 μg/ml) was defined according to the 2016 CLSI standards (42). Multidrug resistance was defined as nonsusceptibility to penicillin combined with resistance to $\geq$2 non-β-lactam antibiotics (6).

Statistical analysis. The study population was divided into two vaccine periods for the analysis: pre-PCV13 (1994 to 2009) and post-PCV13 (2010 to 2014). Descriptive statistics were used to characterize the study population. Fisher's exact test and the chi-square test for trend were used to compare categorical variables. Continuous variables were analyzed by the Mann-Whitney U test. Odds ratios with 95% confidence intervals were calculated to compare the invasive disease potentials of STs (invasive versus noninvasive) for isolates recovered between 2011 and 2014. A two-tailed *P* value of $\leq$0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics version 22.0.0.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01778-16.

TEXT S1, PDF file, 0.1 MB.

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